The zinc finger protein PtaZFP2 negatively controls stem growth and gene expression responsiveness to external mechanical loads in poplar

Ludovic Martin, Mélanie M. Decourteix, Eric Badel, Stephanie Huguet, Bruno Moulia, Jean-Louis J.-L. Julien, Nathalie Leblanc-Fournier

New Phytologist

The zinc finger protein PtaZFP2 negatively controls stem growth and gene expression responsiveness to external mechanical loads in poplar

Ludovic Martin[1,2], Mélanie Decourteix[1,2], Eric Badel[2,1], Stéphanie Huguet[3], Bruno Moulia[2,1], Jean-Louis Julien[1,2] and Nathalie Leblanc-Fournier[1,2]

[1]Clermont Université, Université Blaise Pascal, UMR547 PIAF, BP 10448, F-63000 Clermont-Ferrand, France; [2]INRA, UMR547 PIAF, F-63100 Clermont-Ferrand, France; [3]Unité de Recherche en Génomique Végétale (URGV), Plateforme Transcriptome, UMR INRA 1165 – Université d'Evry Val d'Essonne – ERL CNRS 8196, 2 rue G. Crémieux, CP 5708, F-91057 Evry Cedex, France

Author for correspondence:
Nathalie Leblanc-Fournier
Tel: +33(0)473407930
Email: nathalie.leblanc@univ-bpclermont.fr

Received: *6 December 2013*
Accepted: *17 February 2014*

New Phytologist (2014) **203:** 168–181
doi: 10.1111/nph.12781

Key words: abiotic stimulus, acclimation, mechanical load, poplar (*Populus tremula* × *P. alba*), thigmomorphogenesis, tree, wood, zinc finger transcription factor.

Summary

- Mechanical cues are essential signals regulating plant growth and development. In response to wind, trees develop a thigmomorphogenetic response characterized by a reduction in longitudinal growth, an increase in diameter growth, and changes in mechanical properties. The molecular mechanisms behind these processes are poorly understood. In poplar, *PtaZFP2*, a C2H2 transcription factor, is rapidly up-regulated after stem bending.
- To investigate the function of PtaZFP2, we analyzed *PtaZFP2*-overexpressing poplars (*Populus tremula* × *Populus alba*). To unravel the genes downstream PtaZFP2, a transcriptomic analysis was performed.
- *PtaZFP2*-overexpressing poplars showed longitudinal and cambial growth reductions together with an increase in the tangent and hardening plastic moduli. The regulation level of mechanoresponsive genes was much weaker after stem bending in *PtaZFP2*-overexpressing poplars than in wild-type plants, showing that PtaZFP2 negatively modulates plant responsiveness to mechanical stimulation. Microarray analysis revealed a high proportion of down-regulated genes in *PtaZFP2*-overexpressing poplars. Among these genes, several were also shown to be regulated by mechanical stimulation.
- Our results confirmed the important role of PtaZFP2 during plant acclimation to mechanical load, in particular through a negative control of plant molecular responsiveness. This desensitization process could modulate the amplitude and duration of the plant response during recurrent stimuli.

Introduction

In their natural environment, plants undergo continuous exposure to various mechanical signals such as stresses and strains (Moulia *et al.*, 2011; Hamant, 2013). These mechanical cues are produced intrinsically during tissue or cellular expansion (Ingber, 2005; Hamant *et al.*, 2008) or are triggered by environmental mechanical loads mainly as a result of wind (Moulia *et al.*, 2011). As for other abiotic factors, plants perceive these stimuli and trigger a network of signaling events, resulting in a generic syndrome of growth responses (Potters *et al.*, 2007, 2009). In the case of external mechanical loads such as wind, this syndrome has been called thigmomorphogenesis (Boyer, 1967; Jaffe, 1973). In woody species, the thigmomorphological response of the stem is generally characterized by a reduction in stem elongation (Telewski & Pruyn, 1998; Anten *et al.*, 2005; Coutand *et al.*, 2008; Voelker *et al.*, 2011), a local stimulation of radial growth (Telewski & Pruyn, 1998; Pruyn *et al.*, 2000; Coutand *et al.*, 2009) and a modification of the mechanical properties of the stem (Telewski & Jaffe, 1986; Kern *et al.*, 2005), thereby reducing their exposure and increasing their resistance to wind loads. To study early kinetics of plant growth responses to mechanical loadings, continuous monitoring of primary and secondary growth has been previously carried out in tomato and poplar, respectively (Coutand *et al.*, 2000, 2009). In those two species, a single transient bending was sufficient to induce a complete growth arrest for a few hours. In tomato, the longitudinal growth continued to be reduced for up to 24 h after stem bending, depending on the amount of mechanical strain induced in the tissues (Coutand & Moulia, 2000). By contrast, local diameter growth in poplar stopped for 4 h and then increased for 3–5 d before plant growth went back to a normal rate (Coutand *et al.*, 2009).

In natural conditions, wind induces repeated bending at various frequencies (Rodriguez *et al.*, 2008). In temperate climates, windy and still periods alternate on a typical timescale of several days (Stull, 1988). The effect of daily recurring mechanical loads

on poplar diameter growth has thus been studied (Martin *et al.*, 2010). When one bending was followed by another 24 h later, the magnitude of molecular and growth responses observed after the second bending were lower than those observed after a single bending. About 7 d without any bending were necessary to recover full mechanosensitivity. This accommodation of the mechanosensitivity is believed to be crucial to avoid overresponse to wind (Moulia *et al.*, 2011).

Mechanosensitive responses are controlled by a complex network of regulatory genes. Based on analyses performed on different species, a tentative general flow chart of physiological and molecular responses to mechanoperception has been set up (Telewski, 2006). Two classes of putative mechanosensors have been proposed: mechanosensitive channels and receptor-like kinases inserted into the cell wall–plasma membrane–cytoskeleton network (Monshausen & Gilroy, 2009; Monshausen & Haswell, 2013). Yet the signal transduction pathway leading to growth responses is not clearly understood and the mechanosensors have not been identified so far. Transcriptional analysis conducted on *Arabidopsis* 30 min after a touch stimulus on rosette leaves showed that the expression of up to 700 genes was rapidly modified (Lee *et al.*, 2005). Among the induced genes were the previously described *TOUCH* genes encoding proteins involved either in calcium binding (Braam & Davis, 1990; Sistrunk *et al.*, 1994) or cell wall modifications (Xu *et al.*, 1995). Genes encoding protein kinases, disease resistance protein and transcription factors were also widely represented (Lee *et al.*, 2005). Among these transcription factors, *ZAT10* and *ZAT12*, two genes encoding Q-type C2H2 zinc finger proteins (ZFP) belonged to the 15 most highly touch induced genes.

With their large dimensions, trees are among the most highly exposed plant organisms to environmental mechanical stresses such as wind. Thigmomorphogenesis is crucial to their mechanical stability and longevity (Moulia *et al.*, 2006). Yet, in trees, the molecular events occurring after the application of external mechanical loads are even less well elucidated than in herbs. Studies on *Juglans regia* and *Populus tremula* × *Populus alba* revealed rapid and local induction of expression of *JrZFP2* and *PtaZFP2*, two close homologs of *ZAT12*, after a transient stem bending (Leblanc-Fournier *et al.*, 2008; Martin *et al.*, 2009). Interestingly, the abundance of *PtaZFP2* transcripts was linearly correlated with the amount of mechanical strain induced in the tissues (Coutand *et al.*, 2009; Moulia *et al.*, 2011). Furthermore, repeated stem bending differentially regulated the expression of *PtaZFP2*, its induction level being highly reduced after the second bending (Martin *et al.*, 2010). These observations suggest that *PtaZFP2* may play a key role in the cascade of mechanical signal transduction.

PtaZFP2 belongs to the Q-type C2H2 zinc finger proteins, which represent a large family of eukaryotic transcription factors (Englbrecht *et al.*, 2004; Gourcilleau *et al.*, 2011). The PtaZFP2 amino acid sequence contains all the structural features well characterized in Q-type C2H2 proteins (Martin *et al.*, 2009): two canonical C2H2 zinc fingers (ZF) containing the invariant QALGGH motif essential for their DNA-binding activity (Kubo *et al.*, 1998) and an Ethylene responsive element binding factor-associated Amphiphilic Repression (EAR) motif (Ohta *et al.*, 2001). In *Arabidopsis*, transcription factors containing this repression motif were reported to play important roles in modulating plant growth, development and response to biotic and abiotic stresses (Ohta *et al.*, 2001; Kazan, 2006; Ciftci-Yilmaz & Mittler, 2008). *Arabidopsis* transgenic plants overexpressing different isoforms of C2H2 transcription factors were more tolerant to various abiotic stresses, such as high light, salt, oxidative stress and cold (Rizhsky *et al.*, 2004; Davletova *et al.*, 2005; Vogel *et al.*, 2005; Ciftci-Yilmaz *et al.*, 2007). Interestingly, *ZAT12*, an early cold-responsive gene, is involved in a negative feedback control of the CBF (C-repeat Binding Factor) regulon in the cold transduction pathway (Vogel *et al.*, 2005). Because of the presence of the EAR repression motif in the PtaZFP2 protein sequence, these data suggest a putative involvement of PtaZFP2 in a negative control during the mechanical signaling pathway. In *Arabidopsis*, the roles of the Q-type C2H2 ZFP in plant responses to mechanical loads have been poorly studied. In poplar, such proteins have not yet been functionally characterized.

We hypothesized that the *in vivo* function of PtaZFP2 in a plant submitted to mechanical loads was dual. It could control the growth reduction, but at the same time, it could repress genes involved in the mechanotransduction pathway, thereby inducing a negative control on the mechanosensitivity of the plant to subsequent mechanical loads. To assess these two hypotheses, transgenic poplars overexpressing *PtaZFP2* were produced. As several authors have previously reported the difficulty of generating *Arabidopsis* transgenic plants constitutively over- or underexpressing Q-type C2H2 genes (Sakamoto *et al.*, 2004; Devaiah *et al.*, 2007), we investigated the *in vivo* function of PtaZFP2 by fusing the *PtaZFP2* coding sequence to an inducible promoter activated by 17β-estradiol (Zuo *et al.*, 2000). In this study, we report that overexpression of *PtaZFP2* mimics part of the wind-induced physiological responses. Microarray analysis revealed that the expression of 195 genes is modified in transgenic poplars. A weaker bending-dependent induction of various mechanoresponsive genes was also observed. Altogether, our results suggest that PtaZFP2 not only is involved in the transduction pathway leading to changes in poplar growth, but is also part of a negative regulatory circuit controlling poplar responsiveness to mechanical loads.

Materials and Methods

Vector construction and plant transformation

The estrogen-inducible *LexA-46::PtaZFP2* construct (Supporting Information, Fig. S1) was assembled by subcloning the full-length *PtaZFP2* cDNA (Martin *et al.*, 2009) into the PMDC7 vector using the GATEWAY technology (Invitrogen). This plasmid is a derivative of pER8 (Zuo *et al.*, 2000), which was made GATEWAY-compatible by Curtis & Grossniklaus (2003). The construct was introduced into poplar (*P. tremula* × *P. alba* cv 717-1B4) by *Agrobacterium tumefaciens*-mediated transformation on internodal stem explants cut from *in vitro* plantlets as described by Leplé *et al.* (1992). Transgene orientation and

integrity were checked by DNA sequencing (MilleGen, Labège, France).

Plant material, culture conditions and 17β-estradiol treatments

Transgenic and wild-type (WT) poplars (*P. tremula* × *P. alba* cv 717-1B4) were obtained by *in vitro* micropropagation on MS medium (Murashige & Skoog, 1962). After acclimation, plants were grown in liquid nutrient solution (Morizet & Mingeau, 1976) in a growth chamber at 22°C, with a relative air humidity of 60% and a 16 : 8 h, light : dark cycle with photosynthetic active radiation (PAR) of 50 μmol m^{-2} s^{-1}.

Transgene expression was induced by adding fresh stock solutions of 17β-estradiol directly to the nutrient solution. 17β-estradiol was prepared as a 10 mM fresh stock solution in dimethyl sulfoxide (DMSO). The same treatments were applied to WT poplars. The untreated condition corresponded to plants treated with the same volume of DMSO.

Growth measurements and mechanical tests were carried out on 4-month-old poplars. The 17β-estradiol treatment was started on 2-month-old trees, which were 20 cm (± 1) high and 2.77 mm (± 0.1) wide, and lasted for 2 months. The molecular analyses were conducted on 3-month-old poplars which were 35 cm high and 5.37 mm (± 0.3) wide on average. The treatment duration varied between 1 and 144 h.

Bending treatments

Poplars were set vertically and fixed on the bending device as described in Coutand *et al.* (2009). Plants were left undisturbed for 5 d so that they could settle down after the uncontrolled mechanical disturbance from the installation. Each stem basal part was bent against a plastic tube allowing a homogeneous bending curvature. The tube diameter was chosen depending on each stem diameter, thus imposing a controlled amount of flexural strain (Coutand *et al.*, 2009).

Growth measurements and mechanical behavior

Plant longitudinal growth was determined by measuring stem length from the shoot apex to the stem base. The stem diameter at 25 cm above the collar was measured using a caliper.

Both the elastic (reversible) and plastic (irreversible) behaviors of the wood were characterized on 20-cm-long fresh stem segments. The diameter was measured with a laser beam micrometer. Three-point bending tests were performed using a mechanical testing machine (Instron 5565; Instron, Norwood, MA, USA). The displacement limit was fixed in such a way that the sample experienced elastic and large plastic deformations. The elastic limit separating elastic and plastic behavior was characterized as the minimum strain, ε_{el}, that generates a permanent plastic deformation. The elastic behavior of the tissue (for strains below ε_{el}) was characterized by the longitudinal Young's modulus E. The plastic behavior of the tissue above ε_{el} was characterized by the tangent modulus (E_T) and by the hardening modulus (H). Details of the analysis of the load–displacement curves are given in Fig. S2. Statistical significance was determined by Student's t-test.

Histological analysis

Histological observations were made on the poplars used for growth measurements. Small pieces of stem were cut in a zone above the initial height of the plants (30 cm), and were therefore formed during 17β-estradiol treatment. They were fixed, dehydrated and gradually infiltrated with medium-grade LR White resin (Sigma Aldrich) according to Azri *et al.* (2009). Semithin sections were stained with toluidine blue, dried, mounted in Eukitt and examined under a Zeiss Axioplan 2 microscope. Data were recorded using a digital camera (AxioCam HR; Zeiss) and image analysis was undertaken using the ImageJ software (Schneider *et al.*, 2012). After image segmentation, cell walls and lumens were segregated. The ratio between the cumulated lumen surface and the total surface was referred to as the 'vessel lumen fraction'. The ratio between the cumulated cell wall surface and the total surface was referred to as the 'cell wall fraction'. Statistical significance was determined by Student's t-test.

RNA isolation and cDNA synthesis

For the real-time quantitative reverse transcription polymerase chain reaction (RT-PCR) experiments, RNAs were extracted from 150 mg of bent stems using CTAB extraction buffer (Chang *et al.*, 1993), and treated with RNase-free RQ1 DNase (Promega). RNA was quantified spectrophotometrically and checked by agarose gel electrophoresis. First-strand cDNA was synthesized from 1 μg total RNA using oligodT and SuperScript III (Invitrogen).

For the microarray experiment, RNA was extracted from plants preliminarily treated with 10 μM 17β-estradiol for 96 h, in order to attain a *PtaZFP2* transcript accumulation similar to the one observed after a stem bending (Fig. 1c). RNA was isolated from 100 mg of bent stem using a Rneasy Plant Mini kit followed by a DNAse1 treatment (Qiagen). Before labeling, RNA was quantified using the RiboGreen RNA Quantification Reagent (Invitrogen) and integrity was checked with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbroon, Germany).

Microarray analysis

Three independent biological experiments were analyzed by Affymetrix (Santa Clara, CA, USA) GeneChip Poplar Genome Array oligonucleotide microarrays. For each genotype (WT and *PtaZFP2*-OE line #39), each independent experiment was hybridized with complementary RNA obtained from 2 μg of total RNA made of a pool from two individuals. The amplification, labeling, hybridization, and imaging procedures were performed at the URGV Transcriptomic Plateform (Evry, France) according to the manufacturer's instructions (Affymetrix, http://www.affymetrix.com). Arrays were scanned with the GeneChip Scanner 3000-7G piloted by the GeneChip Operating Software

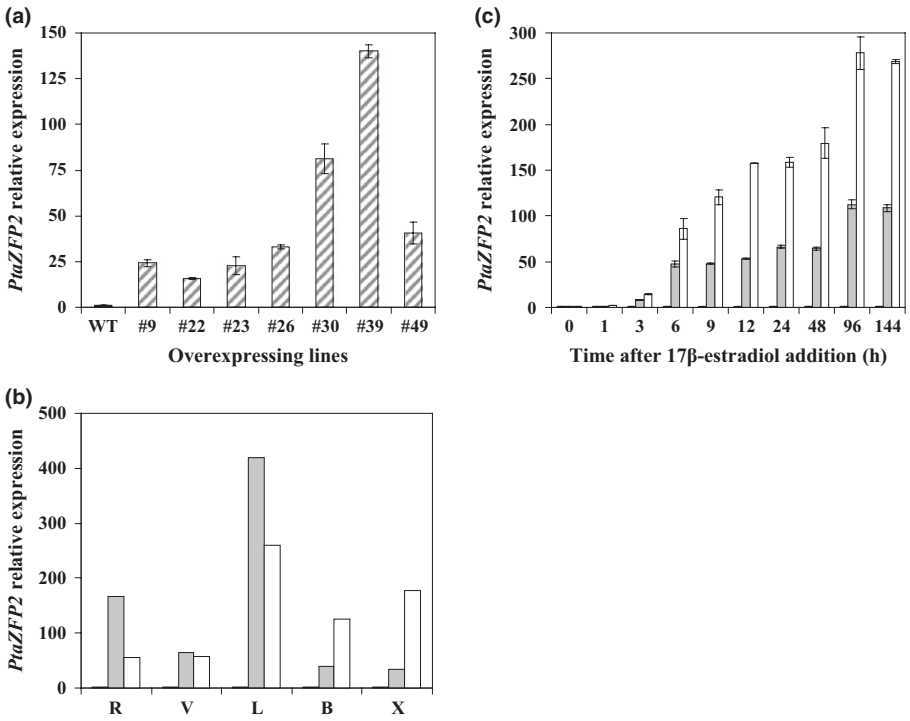

Fig. 1 Molecular characterization of *PtaZFP2*-overexpressing (*PtaZFP2*-OE) poplars (*Populus tremula* × *Populus alba*) by real-time PCR. (a) Expression level of *PtaZFP2* in the seven transgenic single copy lines (#9 to #49) and wild-type (WT) poplars 24 h after transgene activation with 5 μM of 17β-estradiol. (b) Tissue localization of *PtaZFP2* transcript induction was performed by separately harvesting roots (R), veins (V) and lamina (L) from poplar leaves, stem peeled bark (containing bark, phloem, and cambial zone; B) and debarked stem (containing differentiating xylem, mature xylem, and pith; X) of WT (black bars) and *PtaZFP2*-OE lines (#30 (gray bars) and #39 (white bars)) 24 h after 10 μM 17β-estradiol treatment. (c) Time course of transgene activation by 17β-estradiol (10 μM) in stem in lines #30 (gray bars), #39 (white bars) and the WT (black bars). *EF-1α* transcript abundance was used as a reference. Relative transcript abundance was determined by comparing the *PtaZFP2* expression level of treated and untreated plants of each line with WT untreated plants and corresponds to the means ± SE of two independent experiments (a, c) or one independent experiment (b).

(GCOS). Raw data were normalized using the GC-RMA algorithm (Irizarry *et al.*, 2003).

To determine which genes were differentially expressed between WT and *PtaZFP2*-OE line #39, a two-group *t*-test assuming equal variance between groups was performed. To fit the assumption of equal variance of gene expression between groups, genes displaying extreme variation were excluded from the analysis. The raw *P*-values were adjusted by the Bonferroni method (Ge *et al.*, 2003). A gene was declared differentially expressed if the Bonferroni *P*-value was below 0.05. Microarray raw, normalized data and further details of the samples are available through both the CATdb database (AFFY_POP_2011_08_POPLAR ESTRADIOL STUDY) and the Gene Expression Omnibus repository at the National Center for Biotechnology Information (GEO submission GSE43533; http://www.ncbi.nlm.nih.gov/geo/). Enrichment of gene ontology (GO) terms was evaluated with the AgriGO SEA tool (Singular Enrichment Analysis) against the 'Populus Affymetrix Genome Array' as a background, using default parameters with the exception of the use of the Bonferroni multi-test adjustment for multiple comparisons (significance level 0.05) (http://bioinfo.cau.edu.cn/agriGO/analysis.php). For the analysis conducted on up-regulated genes, the 'minimum number of mapping entries' was set to 4. The enrichment was also tested using The Arabidopsis Information Resource (TAIR) Gene Ontology annotation search tool (http://www.arabidopsis.org/tools/bulk/go/) with the *Arabidopsis* matches of the differentially expressed poplar genes.

Real-time quantitative RT-PCR experiments

Real-time RT-PCR amplifications were carried out using an iCycler IQ (Bio-Rad). Each PCR reaction (15 μl) contained 2 μl of 1 : 40 dilution of the first cDNA strands, primers (0.3 μM of each), and MESA GREEN qPCR MasterMix Plus (Eurogentec, Seraing, Belgium). After a heat step at 94°C for 5 min, PCR conditions were as follows: 40 cycles consisting of denaturing (94°C, 10 s), annealing (60°C, 10 s) and elongation (72°C, 15 s), ending with a final elongation step at 72°C for 5 min.

Elongation factor-1α (*EF-1α*) was retained as the reference gene (Martin *et al.*, 2009). The specificity of the primers (listed in Table S1) was checked by sequencing the PCR products (Beckman Coulter Genomics, Takeley, UK).

The relative quantitative abundance (Q_r) was calculated by comparison with the expression of *EF-1α* using the delta–delta method mathematical model (McMaugh & Lyon, 2003). The specificity was confirmed by determining the melt curves for the PCR products and by gel electrophoresis. Real-time PCR amplifications were carried out in triplicate on at least three independent experiments. A Kruskal–Wallis test was used to determine

overall statistical significance. Statistically different groups were obtained with a Newman–Keuls test.

Results

Production of transgenic poplars overexpressing *PtaZFP2* in physiological amounts

Among the 24 *PtaZFP2*-overexpressing lines (*PtaZFP2*-OE) recovered from independent transformation events, seven contained a single copy of the transgene (lines #9, #22, #23, #26, #30, #39 and #49; e.g. Fig. S1). The *PtaZFP2* gene expression was tested 24 h after the addition of 5 μM of 17β-estradiol in nutrient solution, corresponding to the optimal 17β-estradiol concentration used in *Arabidopsis* (Zuo *et al.*, 2000). As shown in Fig. 1(a), *PtaZFP2* expression was 15–140 times higher in *PtaZFP2*-OE lines than in WT poplars grown under controlled conditions. In the absence of an inducer, the *PtaZFP2* expression level of *PtaZFP2*-OE lines was identical to that of WT plants (Fig. S1). In WT poplars, 17β-estradiol treatment had no effect on the expression of the *PtaZFP2* endogenous gene (Fig. S1). Under these conditions, #30 and #39 *PtaZFP2*-OE lines exhibited levels of *PtaZFP2* expression comparable to that observed in WT poplars after a transient stem bending (Martin *et al.*, 2010), and were selected for further phenotypic characterization. For these two lines, the maximum induction was observed for 10 μM of 17β-estradiol, higher concentrations having no additive effects (data not shown). This concentration was then used for all experiments.

As this type of inducible construction had never been used in poplar before, we examined changes in the abundance of *PtaZFP2* transcripts in different organs 24 h after 17β-estradiol addition in #30 and #39 *PtaZFP2*-OE lines. As shown in Fig. 1(b), *PtaZFP2* overexpression was observed in all organs tested, indicating that the inducible system had been activated in the whole plant. For both *PtaZFP2*-OE lines, the highest overexpression was observed in leaves, probably resulting from the accumulation of 17β-estradiol through the xylem flow. However, the overexpression found in the lamina showed that 17β-estradiol was not confined to vascular tissues. A significant overexpression of *PtaZFP2* was also observed in both the bark and xylem. In stems, the time course accumulation of *PtaZFP2* transcripts after addition of 17β-estradiol was also analyzed. Similar transcript induction profiles were observed in the treated #30 and #39 *PtaZFP2*-OE lines (Fig. 1c). In these lines, *PtaZFP2* expression was detectable 3 h after the treatment and rose to a peak at 4 d after induction. While in *Arabidopsis* the transgene expression decreased after 96 h (Zuo *et al.*, 2000), in *PtaZFP2*-OE poplar lines it reached a plateau at 96 h, then remaining steady.

Overexpression of *PtaZFP2* induces diminution of poplar growth and modifications of stem histological and mechanical properties

To examine whether *PtaZFP2* overexpression affects poplar growth and wood mechanical properties, *PtaZFP2*-OE and WT poplars were treated for a period of 2 months with 10 μM 17β-estradiol (Fig. 2a). *PtaZFP2* overexpression led to a 15%

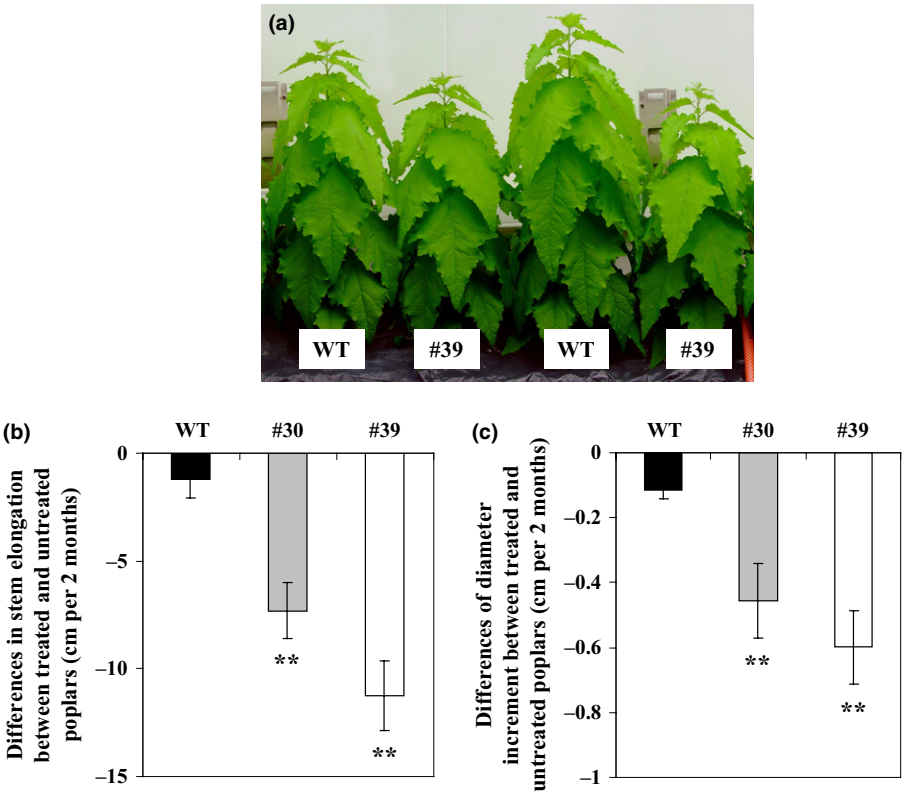

Fig. 2 Growth phenotype of *PtaZFP2*-overexpressing (*PtaZFP2*-OE) and wild-type (WT) poplars (*Populus tremula* × *Populus alba*) treated for 2 months with 17β-estradiol (10 μM). (a) Photographs of 4-month-old WT and *PtaZFP2*-OE line (#39) treated for the last 2 months with 17β-estradiol. (b, c) Effect of 17β-estradiol treatment on stem elongation (b) and stem diameter increment (c) in *PtaZFP2*-OE (#30 and #39) and WT lines compared with untreated plants. Represented values are means of eight to 14 independent plants for each line and treatment ± SE. Statistically significant differences between treated and untreated plants in each line (as determined by *t*-test): **, $P < 0.01$.

reduction of longitudinal growth (Fig. 2b) and an 8% reduction of diameter growth (Fig. 2c), whereas 17β-estradiol had no significant effect on WT plants. This growth reduction was higher in the #39 line than in #30 and seemed to be correlated with that of *PtaZFP2* expression.

In comparison with untreated transgenic plants, the 17β-estradiol-treated #39 line had a significantly higher Young's modulus (+12%, $P = 0.049$) (Table 1). In the plastic regime, they also displayed a significantly higher E_T (+33.3%, $P = 0.018$) and H (+79.4%, $P = 0.031$), so that their tissues were less prone to plastic irreversible deformations under large mechanical loads (plastically stiffer). However, the elastic strain limit ε_{el} was not significantly affected by the treatment. None of these mechanical properties were modified in WT plants after addition of 17β-estradiol (data not shown). To test whether the Young's modulus modification was the result of changes in the wood anatomy, xylem characteristics were measured in stem cross-sections. Overexpression of *PtaZFP2* induced a significant increase (+15%) of the vessel lumen fraction. This was mainly the result of the formation of bigger vessels (Table 2). However, the cell wall fraction was not significantly affected by the treatment. Therefore the previously reported changes in mechanical properties can be attributed to rheological changes of the cell walls. These results indicated that overexpression of *PtaZFP2* modified poplar growth as well as wood anatomy and cell wall mechanical properties, especially in the range of large plastic strains.

Poplar sensitivity to bending is modified by *PtaZFP2* overexpression

We have previously shown that, after a first bending, poplars become less sensitive to subsequent ones (Martin *et al.*, 2010). To evaluate the involvement of *PtaZFP2* overexpression on plant sensitivity to mechanical loads, we compared the transcriptional patterns of four mechanoresponsive genes in WT and *PtaZFP2*-OE #30 and #39 lines treated for 96 h with 17β-estradiol (i.e. when the amount of *PtaZFP2* transcript in *PtaZFP2*-OE is similar to that observed in the WT after a single stem bending). Two of these genes (*PtaTCH4* and *PtaXET6*) encode xyloglucan endotransglycosylases/hydrolases shown to be regulated by mechanical loads in both *Arabidopsis* and poplar (Lee *et al.*, 2005; Martin *et al.*, 2010). The other two are *PtaZFP2* and its closest Q-type C2H2 homolog, *PtaZFP1* (Gourcilleau *et al.*, 2011).

Table 2 Xylem characteristics of *PtaZFP2*-overexpressing (*PtaZFP2*-OE) poplars (*Populus tremula* × *Populus alba*) (#39 line) treated or not with 10 μM of 17β-estradiol for 2 months

Xylem traits	Untreated #39 *PtaZFP2*-OE	17β-estradiol-treated #39 *PtaZFP2*-OE	*P*-value
Wall fraction (%)	69.43 ± 0.64	67.23 ± 0.88	0.101
Vessel lumen fraction (%)	**20.94 ± 0.40**	**24.14 ± 0.35**	**0.004**
Vessel density (no. of vessels mm^{-2})	141 ± 3.17	149 ± 1.8	0.129
Mean vessel lumen area (μm^2)	**1480 ± 11**	**1625 ± 25**	**0.001**

Data are presented as mean ± SE of five plants per treatment. *P*-values (*t*-test) are indicated. Values in bold were significantly different ($P < 0.001$) from controls.

As shown in Fig. 3(a–c), when no bending was applied, no or little basal expression of *PtaTCH4*, *PtaXET6* and *PtaZFP1* was observed in stems of WT and transgenic lines previously treated with 17β-estradiol. As expected, a high transcript accumulation was observed 30 min after stem bending in WT plants for these three genes. By contrast, the increase in mRNA levels after bending was much reduced in *PtaZFP2*-OE lines, although a quantitatively identical mechanical stimulus was applied. These results suggest that the overexpression of *PtaZFP2* triggers a reduction of poplar molecular responses to bending.

Consistent with what was shown previously, when no bending was applied, *PtaZFP2* transcript accumulation was 40 and 90 times higher in *PtaZFP2*-OE #30 and #39 lines, respectively, than in WT plants (Fig. 3d). After bending in *PtaZFP2*-OE lines, a weak increment in *PtaZFP2* expression was detected, probably corresponding to the mechano-induced expression of the endogenous *PtaZFP2* gene. However, the induction level of endogenous *PtaZFP2* gene expression was much lower in *PtaZFP2*-OE lines than that observed in bent WT plants. These observations strongly support the hypothesis that under mechanical stress conditions, PtaZFP2 negatively modulates, indirectly or directly, its own expression and the expression of several mechanoresponsive genes.

Transcriptome profiling of poplar plants overexpressing *PtaZFP2*

To unravel the transcriptional network operating downstream of PtaZFP2, we compared the expression profile of *PtaZFP2*-OE

Table 1 Stem mechanical properties of *PtaZFP2*-overexpressing (*PtaZFP2*-OE) poplars (*Populus tremula* × *Populus alba*) (#39 line) treated or not with 10 μM of 17β-estradiol for 2 months

Mechanical traits	Untreated #39 *PtaZFP2*-OE	17β-estradiol-treated #39 *PtaZFP2*-OE	*P*-value
Young modulus (Mpa)	**1920 ± 88**	**2150 ± 58**	**0.049**
Elastic limit	$7.71 \times 10^{-3} \pm 0.29 \times 10^{-3}$	$6.89 \times 10^{-3} \pm 0.33 \times 10^{-3}$	0.082
Tangent modulus (Mpa)	**1025 ± 56**	**1366 ± 111**	**0.018**
Hardening modulus (Mpa)	**2333 ± 294**	**4185 ± 700**	**0.031**

Data are presented as means ± SE of seven plants per treatment. *P*-values (*t*-test) are indicated. Values in bold were significantly different ($P < 0.05$) from controls.

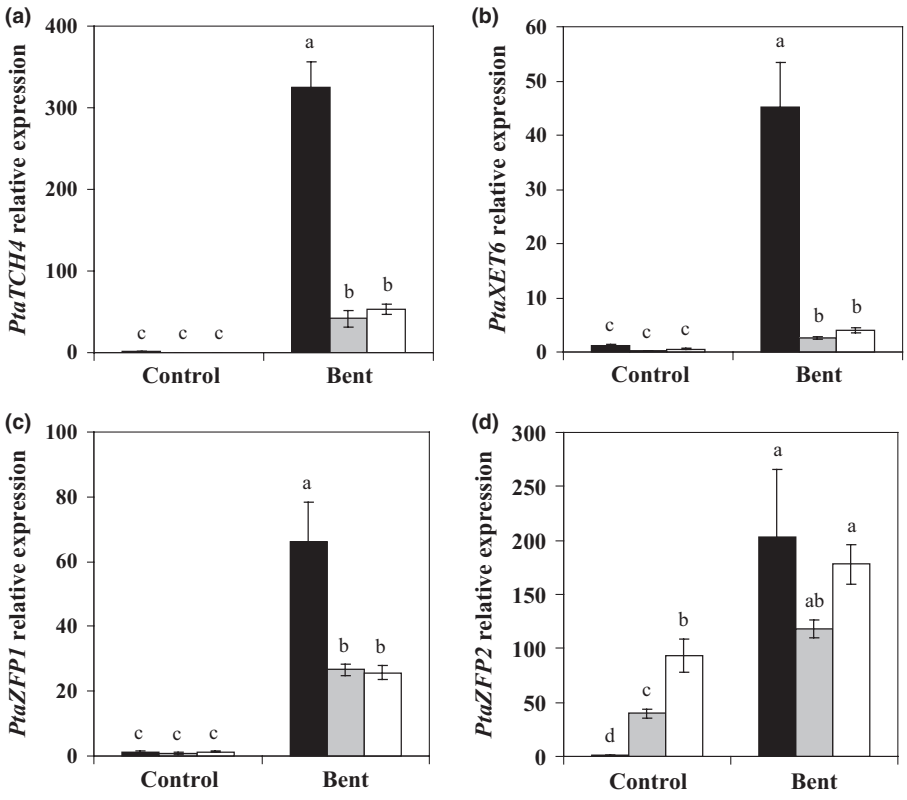

Fig. 3 Molecular assessment of *PtaZFP2*-overexpressing (*PtaZFP2*-OE) poplars' sensitivity to bending. Expression of four mechanosensitive genes, *PtaTCH4* (a), *PtaXET6* (b), *PtaZFP1* (c), and *PtaZFP2* (d), was monitored by real-time PCR (qPCR) in stems of *Populus tremula* × *Populus alba* wild-type (WT; black bars) and *PtaZFP2*-OE lines (#30 (gray bars) and #39 (white bars)). Plants were treated with 17β-estradiol (10 μM) for 4 d and stems were collected 30 min after a single transient bending (Bent) or without any stimulation (Control). In each line and condition, the relative transcript abundance was determined by comparison with the gene expression level in 17β-estradiol-treated WT plants using *EF-1α* transcript abundance as a reference. Represented values are means of three to seven biological replicates ± SE. Significant differences ($P < 0.01$) of responses are indicated by different letters.

lines treated with 17β-estradiol for 96 h with that of WT plants. In the *PtaZFP2*-OE plants, a total of 132 genes matching 138 probesets were down-regulated and a total of 63 genes matching 76 probesets were up-regulated (Table S2). This disproportion between down- and up-regulated genes points towards PtaZFP2 being mainly part of a negative regulatory pathway.

In order to identify over-represented gene categories in our data set when compared with their proportion on the Affymetrix GeneChip Poplar Genome Array, we explored the functional categorization of the deregulated genes using the AgriGO SEA tool (Singular Enrichment Analysis). Up-regulated genes belonged to two major over-represented categories: 'sequence-specific DNA binding' and 'response to biotic stimulus'. The categories 'response to other organism' and 'multi-organism process' were also over-represented, but contained identical genes to the category 'response to biotic stimulus', to which we will solely refer hereafter (Table 3). Among the four genes belonging to the 'sequence-specific DNA binding' category were three WRKYs, a group of transcription factors known for their involvement in senescence regulation and plant defense against both biotic and abiotic stresses (Rushton *et al.*, 2010). Genes down-regulated by *PtaZFP2*-OE belonged to three major over-represented functional categories, namely 'cellular response to chemical stimulus', 'catalytic activity' and 'tetrapyrrole binding'. The group of

Table 3 Over-represented gene ontology (GO) terms in up- and down-regulated genes of *PtaZFP2*-overexpressing (*PtaZFP2*-OE) poplars (*Populus tremula* × *Populus Alba*)

GO term	Description	Number in input list	Number in background[1]	*P*-value	FDR
Up-regulated					
GO:0051707	Response to other organism	5	447	0.000 25	0.011
GO:0009607	Response to biotic stimulus	5	498	0.0004	0.018
GO:0051704	Multi-organism process	5	589	0.000 86	0.039
GO:0043565	Sequence-specific DNA binding	4	272	0.0004	0.0071
Down-regulated					
GO:0070887	Cellular response to chemical stimulus	7	448	0.000 48	0.041
GO:0003824	Catalytic activity	54	12 398	0.0009	0.038
GO:0046906	Tetrapyrrole binding	6	370	0.001	0.043

FDR, false discovery rate.
The table displays significantly over-represented GO categories as determined by AgriGO SEA using a Fisher test and a Bonferroni multi-test correction.
[1]The chosen background is the Populus Affymetrix Genome Array, which consists of 35 906 annotations.

'catalytic activity' genes made up 39% of the down-regulated genes and participated mainly in phosphate (phosphatases and kinases), carbon and fatty acid metabolism. Among the seven genes belonging to the 'cellular response to chemical stimulus' category, five were involved in plant hormonal regulation, especially ethylene and auxin. The over-representation of hormonal-related processes was also evident in the 'catalytic activity' category, as six genes out of 54 were assigned a function in hormone biosynthesis, degradation or transport (Table 3).

The incompleteness of poplar GO annotations prompted us to conduct a second analysis by interrogating the TAIR GO annotation search tool with the *Arabidopsis* matches of the differentially expressed poplar genes. This approach confirmed globally the results obtained with the AgriGO SEA tool (Table S3), highlighting the prevalence of genes responding to various stimuli and stresses. Overall, our GO data are in accordance with the previously identified involvement of C2H2 from other species (e.g. ZAT12 in *Arabidopsis*) in response to biotic and abiotic stimuli (Rizhsky *et al.*, 2004; Vogel *et al.*, 2005; Kilian *et al.*, 2007; Sun *et al.*, 2010; Luo *et al.*, 2012).

PtaZFP2 modulates the expression of various genes involved in mechanotransduction

In WT poplar, a single transient stem bending triggers a rapid induction of *PtaZFP2* expression (Martin *et al.*, 2009; Fig. 3d), suggesting that, besides its role in the desensitization process, this transcription factor might be a short-term inducer of early mechanical responses. To investigate whether the most induced and repressed genes identified in *PtaZFP2*-OE poplars were effectively mechanoresponsive, we compared the expression of 10 genes, 30 min and 2 h after a single transient stem bending in *PtaZFP2*-OE and WT poplars (Figs 4, 5). In *PtaZFP2*-OE poplars, the transcript profiles of the 10 genes characterized by qRT-PCR assays were consistent with those in microarray analysis (Table S2). Apart from a gene encoding a putative alpha-fucosidase (*PtaGDSL*), nine of the 10 genes misregulated in *PtaZFP2*-OE poplars also appeared to be rapidly deregulated by a transient bending in WT plants. The four *PtaZFP2*-down-regulated genes, encoding a Clavata-like peptide (*PtaCLE1-like*), a peroxidase (*PtaPO2*), a putative auxin ABC transporter (*PtaABC*), an ethylene receptor (*PtaETR1*), were rapidly repressed by bending in WT poplars (Fig. 4). Among the *PtaZFP2*-up-regulated genes, two different expression profiles could be distinguished. The basal expression level of genes encoding an alpha-amylase (*PtaAMY1*), a transmembrane protein kinase (*PtaFERONIA*), a calmodulin-like protein (*PtaCML42*) and an 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (*PtaACO4*) was higher in *PtaZFP2*-OE plants than in WT plants without bending, but lower than the mRNA accumulation observed in WT plants after bending (Fig. 5). Considering *PtaNIMIN-1*, a gene involved in the regulation of the pathogen defense in *Arabidopsis* (Hermann *et al.*, 2013), its expression is up-regulated by stem bending, but the basal level detected in *PtaZFP2*-OE plants is higher than the expression level observed in WT poplars after bending (Fig. 5).

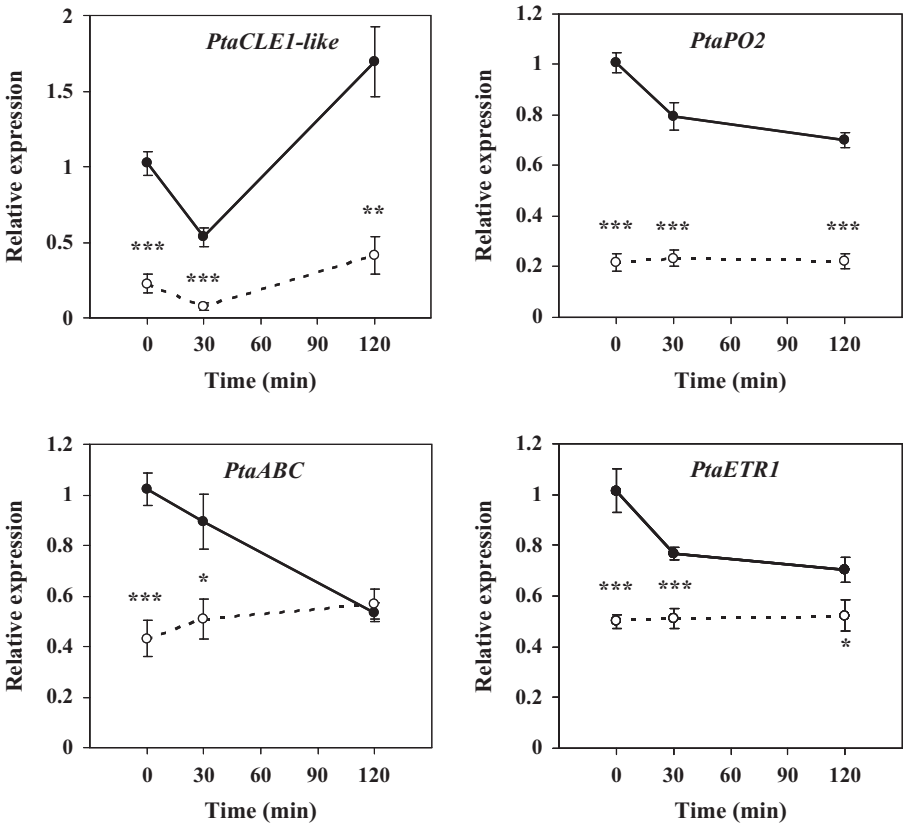

Fig. 4 Expression levels of four genes down-regulated in *PtaZFP2*-overexpressing (*PtaZFP2*-OE) poplars (#39) without or after a transient stem bending. *PtaZFP2* expression was monitored by real-time PCR (qPCR). Plants (*Populus tremula* × *Populus alba*) were treated with 17β-estradiol (10 μM) for 4 d. The bent zone of stems was collected 30 and 120 min after a single transient bending. In each line and condition, the relative transcript abundance was determined by comparison with the gene expression level in 17β-estradiol-treated wild-type (WT) plants using *EF-1α* transcript abundance as a reference. Closed circles, WT; open circles, #39. Represented values are means of five to seven biological replicates ± SE. Asterisks indicate statistically significant differences (Student's *t*-test) between WT and *PtaZFP2*-OE poplars: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

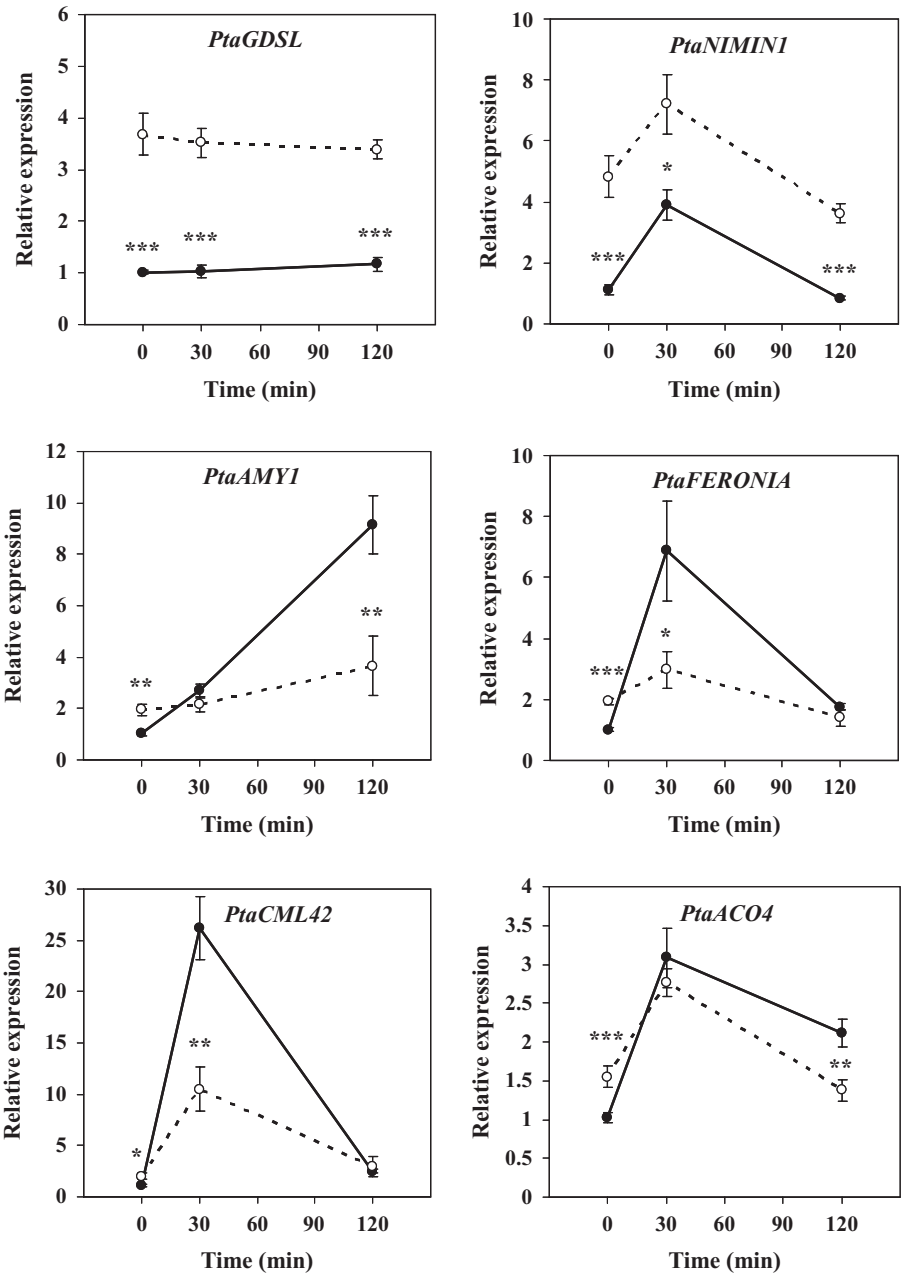

Fig. 5 Expression levels of six genes up-regulated in *PtaZFP2*-overexpressing (*PtaZFP2*-OE) poplars (#39) without or after a transient stem bending. *PtaZFP2* expression was monitored by real-time quantitative PCR (qPCR). Plants (*Populus tremula* × *Populus alba*) were treated with 17β-estradiol (10 μM) for 4 d and the bent zone of the stems was collected 30 and 120 min after a single transient bending. In each line and condition, the relative transcript abundance was determined by comparison with the gene expression level in 17β-estradiol-treated wild-type (WT) plants using *EF-1α* transcript abundance as a reference. Closed circles, WT; open circles, #39. Represented values are means of five to seven biological replicates ± SE. Statistically significant differences (Student's *t*-test) between WT and *PtaZFP2*-OE poplars: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Of particular interest, the regulation by bending of these nine mechanoresponsive genes appeared to be strongly reduced in *PtaZFP2*-OE poplars (Figs 4, 5), revealing again the role of *PtaZFP2* in a desensitization mechanism. Moreover, our results indicate that some of the genes misregulated in *PtaZFP2*-OE poplars are actually early molecular actors of mechanotransduction and that overexpression of *PtaZFP2* mimics part of the short-term effects induced by a transient bending.

To assess whether these conclusions may be conserved across species, we then compared the transcriptome profiling of *PtaZFP2*-OE poplars with an *Arabidopsis* 'touch dataset' obtained from rosettes 30 min after a touch stimulus (Lee *et al.*, 2005). In the *Arabidopsis* 'touch dataset', *ZAT12/RHL41* (AT5G59820), the putative *Arabidopsis* homolog of *PtaZFP2*, was one of the most highly induced genes among the 589 up-regulated ones. Additionally, several *PtaZFP2*-OE deregulated genes were also deregulated in the touched *Arabidopsis* (shown in bold in Table S2). Deregulated genes included *PEPKR1* (Potri.015G136900), *MURUS4* (Potri.001G459700) and *RVE1* (Potri.017G144800) for the *PtaZFP2*-OE down-regulated genes, and Potri.010G25 1000 (uncharacterized protein), an EP3 chitinase encoding gene (Potri.013G125000), *WRKY53* (Potri.014G096200), *PLA2A* (Potri.007G040400), a phytosulfokine two precursor encoding gene (Potri.002G116300), and *WRKY40* (Potri.018G019800) for the *PtaZFP2*-OE up-regulated genes. Interestingly, *WRKY40* was one of the most highly induced genes in the touch dataset. *RVE1* was down-regulated both in our dataset and in the touch dataset. These data suggest that PtaZFP2 and its *Arabidopsis* homolog ZAT12 could regulate part of the transcriptional network triggered by mechanical stimuli.

Discussion

PtaZFP2 role in stem growth

In response to sublethal abiotic stress conditions, herbaceous plants exhibit a generic 'stress-induced morphogenic response' (inhibition of cell elongation, localized stimulation of cell division and alteration in cell differenciation status) that could be part of a general acclimation strategy to diminish plant exposure to stress (Potters *et al.*, 2007). Such morphogenic responses are also described in woody plants after wind exposure (Telewski, 2006). However, the molecular mechanisms triggering this thigmomorphogenetic syndrome have still not been fully elucidated.

Here, we describe the first direct evidence of the role of a C2H2-type zinc finger protein in the control of tree growth and development. The phenotype of the *PtaZFP2*-OE lines cannot be attributed to some indirect deleterious effect, as it was observed in two independent lines with different transgene insertion sites (Fig. S1) and 17β-estradiol had no effect on WT poplar. Furthermore, no physiological effect of the XVE system itself was observed in transgenic *Arabidopsis* plants (Zuo *et al.*, 2000). *PtaZFP2* overexpression led to a 15% reduction in longitudinal growth and an 8% diminution in radial growth (Fig. 2b,c), which is reminiscent of the early growth responses of the thigmomorphogenetic syndrome. Indeed, the first responses to a transitory bending are a cessation of both subapical and cambial growth (Coutand *et al.*, 2000, 2009). In poplar, the 4 h radial growth arrest observed in the bent zone of the stem (Coutand *et al.*, 2009) coincided with the time of local *PtaZFP2* induction (Martin *et al.*, 2009). PtaZFP2 is thus likely to be directly involved in the establishment of the first inhibitory stage of the growth responses to mechanical loads.

One of the recurrent phenotypes in *Arabidopsis* plants overexpressing Q-type C2H2 genes such as *ZAT12* (Vogel *et al.*, 2005), *STZ* (Sakamoto *et al.*, 2004) or *ZAT6* (Devaiah *et al.*, 2007) is growth and development retardation. RNAi suppression of *ZAT6* is lethal (Devaiah *et al.*, 2007), and, interestingly, our attempts to suppress *PtaZFP2* by constitutive RNAi in poplars were also unsuccessful (personal data), again suggesting a key role of such C2H2 transcription factors in regulating plant growth and development. Whereas the impact of these genes on tolerance to various abiotic stresses was well studied in *Arabidopsis*, little is known on how these genes regulate growth responses.

Using transcriptome analysis of *PtaZFP2*-OE plants, PtaZFP2 was found to control directly or indirectly the expression of 195 genes with widespread putative functions. Almost 70% of these genes were down-regulated, which shows PtaZFP2 could be part of a negative regulatory circuit that impedes the response of mechanically responsive genes. Part of the transcriptional network operating downstream PtaZFP2 could explain the growth reduction phenotype. The most highly *PtaZFP2*-OE down-regulated gene *PtaCLE1-like* (Potri.001G376100) matches an A-type peptide of the Clavata3/Endosperm Surrounding Region (ESR)-related (CLE) peptide family. *PtaCLE1-like* is also down-regulated in the stem of WT poplar shortly after mechanical loads (30 min) and up-regulated thereafter (2 h after bending; Fig. 4) Although A-type CLE peptide function in vascular development has been poorly characterized, especially in trees, some A-type CLE peptides were shown to enhance the vascular cell proliferation-stimulating activity of B-type CLE (Etchells & Turner, 2010).

In trees, auxin and ethylene responsiveness is known to control cambial cell activity (Nilsson *et al.*, 2008; Love *et al.*, 2009). In our dataset, the deregulation of many genes related to hormonal signaling, especially ethylene and auxin, was striking. Probesets matching genes involved in auxin response, the auxin response factors *ARF1* and *ARF6*, and the auxin signaling F-box *AFB2*, were down-regulated in the *PtaZFP2*-OE plants. *ABCB15*, a putative auxin transport protein encoding gene, was also down-regulated. Such a negative regulation of auxin responsiveness by C2H2 zinc finger proteins has also been identified in *Arabidospis*, but for distantly related homologs of PtaZFP2 (AZF1 and AZF2), and in other stress conditions (Kodaira *et al.*, 2011).

We also found that many genes related to ethylene biosynthesis and signaling were deregulated by *PtaZFP2* overexpression. For example, *ETR1*, *ERS1*, two of the ethylene receptors, and *EIN3*, the transcription factor on which ethylene signaling is supposed to converge, were down-regulated. This may indicate a decrease in ethylene sensitivity. At the same time, the ACC oxidase gene *ACO4* involved in ethylene biosynthesis was strongly up-regulated. This is not necessarily contradictory, as mutations resulting in hormone insensitivity often lead to a positive feedback on biosynthesis (Vandenbussche *et al.*, 2012).

Overall, these data could make *PtaCLE1-like*, auxin and ethylene responsiveness good candidates to explain part of the decreased radial growth of *PtaZFP2*-OE poplars.

PtaZFP2 affects stem mechanical properties

Mechanical loads modified the mechanical properties of the stems. Multiple stem bending in poplar induced an increase in the cell wall fraction (higher specific density) of the wood but a decrease in its elastic stiffness (Young's modulus) and flexural strength (modulus of rupture). Therefore the specific cell wall properties decreased even more (Pruyn *et al.*, 2000; Kern *et al.*, 2005). In our study, *PtaZFP2*-OE poplars did not show significant changes in the cell wall fraction but they displayed a slightly higher Young's modulus (+11.9%). More dramatic changes occurred regarding plastic properties, with an increase in the tangent modulus E_T (+33.3%, $P = 0.018$) and in the hardening modulus H (+79.4%, $P = 0.031$). This suggests that for large mechanical stresses, the plastic nonrecoverable strain was reduced and the wood of the stem was less flexible. These results reveal changes in the cell wall structure/composition. Plastic hardening is driven by macromolecular slidings. Although the molecular mechanisms controlling wall polymer sliding have not yet been studied in green wood, they probably depend on modifications of cell wall polymer quality/reciprocal arrangements. The changes in plastic hardening could be orchestrated by PtaZFP2, as supported by some of the transcriptional modifications observed in the *PtaZFP2*-OE plants. Indeed, the most highly up-regulated

gene (*PtaGDSL*) in the *PtaZFP2*-OE plants is similar to an *Arabidopsis* alpha-fucosidase (AT3G26430), an enzyme breaking down fucose residues (Del Bem & Vincentz, 2010). Previous studies have suggested that the presence of the L-fucose-containing trisaccharide side-chain on xyloglucan chains is a key component in the association with cellulose (Vanzin *et al.*, 2002). Interestingly, *mur2*, an Arabidopsis mutant almost completely devoid of fucosyl residues on xyloglucans, displayed reduced cell wall strength (Vanzin *et al.*, 2002). Xyloglucans, and especially (fucogalacto) xyloglucans, are key wall hemicelluloses that bind to cellulose microfibrills by hydrogen bonds. In the cell wall, xyloglucan endotransglycosylase/xyloglucan endo-transglycosylase/hydrolase (XET/XTH) specifically catalyze the endolytic cleavage and re-ligation of xyloglucan chains. Cleavage of xyloglucans by XET/XTH would facilitate the sliding while re-ligation would allow the cell wall to return to a stabilized state. In our dataset, genes encoding Pt-XTRA.1 (Potri.003G097300) and Pt-EXT.15 (Potri.014G140300), two XET/XTH proteins similar to the *Arabidopsis* XTR4/XTH30 and XTR5, were up- and down-regulated, respectively. Furthermore, poplars overexpressing *PttXET6-34* tend to have significantly wider vessel elements than the WT (Nishikubo *et al.*, 2011), a phenotype that was also observed in the *PtaZFP2*-OE plants.

Thus, the up-regulation of *Pt-XTRA.1* and *PtaGDSL*-like in response to *PtaZFP2* overexpression may be partly responsible for the *PtaZFP2*-OE mechanical plastic behavior changes and vessel diameter phenotype. But PtaZFP2 does not seem to control the whole thigmomorphogenetic response of wood anatomical and mechanical properties (especially specific density and cell wall elastic stiffness).

As a functional benefit of a higher hardening modulus controlled by PtaZFP2, our results suggest an adaptive process in the wind at the whole-plant level, as the amount of unrecoverable strain after large mechanical loads should be reduced.

PtaZFP2 reduces the induction of molecular responses to bending: a driver of mechanosensitivity?

In poplars, the effects of stem bending on growth and molecular responses depend on the mechanical history of the plant (Martin *et al.*, 2010), with a weaker induction of several mechanoresponsive genes as well as of secondary growth responses after successive bendings (Martin *et al.*, 2010). Desensitization of growth responses was also suggested in experiments on *Ulmus americana*: no increment of the secondary growth response was detected when increasing the number of loadings from five to 80 bendings a day (Telewski & Pruyn, 1998). In *Arabidopsis*, Arteca & Arteca (1999) demonstrated that multiple touch stimulations were progressively less effective in promoting *ACS6* expression, a gene encoding ACC synthase. However, by following early cellular events, such as calcium or pH modification in *Arabidopsis*, a desensitization phenomenon (potentially as a result of a refractory period of the channels) was only observed when intervals between two touch stimulations were smaller than 20–45 s (Knight *et al.*, 1992; Monshausen *et al.*, 2009). Therefore, it is more likely that the long-term desensitization observed in our study involves a change in the amount of mechanoreceptors and/or of key controlling actors of responsiveness rather than being an effect on the kinetic properties of each mechanosensor.

In our work, the expression level of numerous mechanoresponsive genes after a transient stem bending was much weaker in *PtaZFP2*-OE poplars than in the WT (Figs 3–5), showing that PtaZFP2 negatively modulates plant responsiveness to mechanical load. Two hypotheses can be suggested. First, the perception capacities could be modified. Our knowledge of plant mechanosensors is not good enough to evaluate the role of PtaZFP2 in modifying perception capacities. However, two genes, *FERONIA* (Potri.017G097500) and *WAKL2* (Potri.004G192500), encoding transmembrane protein kinases described as putative mechanosensors of wall status (Monshausen & Gilroy, 2009), are up-regulated in *PtaZFP2*-OE plants. The members of the receptor-like kinase (RLK) family play an important role in cell wall integrity surveillance (Humphrey *et al.*, 2007; Cheung & Wu, 2011) and are supposed to feedback-regulate cell wall properties (Guo *et al.*, 2009). Their regulation by PtaZFP2 or external mechanical loads has never been reported before. The link between an increase in their expression and the desensitization process, however, is not direct.

The second hypothesis is that some early components of the mechanotransduction could be modified after the first stimulation, thus limiting the response to subsequent stimulations. The study of PtaZFP2 structural features led to the hypothesis that this protein may act as a transcriptional repressor (Gourcilleau *et al.*, 2011), through the presence of an EAR motif (Ohta *et al.*, 2001; Kazan, 2006). Thus, PtaZFP2 could be directly involved in the desensitization phenomenon, through a negative feedback regulation of the early molecular actors involved after mechanostimulation. Indeed, when we studied the expression of 10 genes derived from the transcriptomic analysis, nine of these genes were also regulated by bending in WT poplars (Figs 4, 5), the majority being regulated as early as 30 min after the mechanical load.

The negative effect of PtaZFP2 on plant responsiveness to bending could also be explained by a less direct mechanism involving PtaZFP2 deregulation of other transcription factors. Interestingly, among the PtaZFP2 up-regulated genes, four genes have been described as negative regulators of plant response to biotic or abiotic stresses in *Arabidopsis*. CML42 is a Ca^{2+} sensor having multiple functions in biotic and abiotic stress responses (Vadassery *et al.*, 2012). *Arabidopsis* WRKY18, -40, and -60 negatively regulated resistance to *Pseudomonas syringae* (Xu *et al.*, 2006). Arabidopsis NIM1-INTERACTING (NIMIN1) suppressed NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) activity, a central regulator of the pathogen defense reaction (Hermann *et al.*, 2013). Furthermore, we demonstrated that these genes are effectively early molecular actors of the mechanical transduction pathway in poplar. *PtaNIMIN-1* (Potri.002G190800) and *PtaCML42* (Potri.006G112500) gene expressions are induced early by mechanical load in poplars (Fig. 5). *PtaWRKY53* (Potri.014G096200) and *PtaWRKY40* (Potri.018G019800) were up-regulated in *PtaZFP2*-OE and also up-regulated in response to touch in *Arabidopsis* (Lee *et al.*, 2005). Thus, after a first mechanical load, PtaZFP2, in concert

with these negatively acting molecular actors, could prevent or reduce the reactivation of the mechanical signaling pathway at the time of a subsequent mechanical load.

This functional study confirmed the important role of *PtaZFP2* during plant acclimation, being involved both in growth rate reduction and plant desensitization to mechanical load. This desensitization process is essential during recurrent mechanical stimulations, as it could modulate the magnitude and duration of the plant response in order to prevent significant costs for reduced plant growth. One challenge will now be to understand how this state of desensitization to mechanical loads could last for several days.

Acknowledgements

This research received support from the French Agence Nationale de la Recherche, grant ANR-09-BLAN-0245-01. We thank Prof. Nam Hai Chua for kindly sending us the PMDC7 vector and Christelle Boisselet, Brigitte Girard and Norbert Frizot for their help with plant production, histological preparations and mechanical tests, respectively.

References

Anten NPR, Casado-Garcia R, Nagashima H. 2005. Effects of mechanical stress and plant density on mechanical characteristics, growth, and lifetime reproduction of tobacco plants. *American Naturalist* **166**: 650–660.

Arteca JM, Arteca RN. 1999. A multi-responsive gene encoding 1-aminocyclopropane-1-carboxylate synthase (ACS6) in mature Arabidopsis leaves. *Plant Molecular Biology* **39**: 209–219.

Azri W, Chambon C, Herbette S, Brunel N, Coutand C, Leplé JC, Ben Rejeb I, Ammar S, Julien JL, Roeckel-Drevet P. 2009. Proteome analysis of apical and basal regions of poplar stems under gravitropic stimulation. *Physiologia Plantarum* **1362**: 193–208.

Boyer N. 1967. Modification de la croissance de la tige de bryone (*Bryonia dioica*) à la suite d'irritations tactiles. *Compte Rendu de l'académie des sciences de Paris* **267**: 2114–2117.

Braam J, Davis RW. 1990. Rain-induced, wind-induced, and touch-induced expression of calmodulin and calmodulin-related genes in *Arabidopsis. Cell* **60**: 357–364.

Chang S, Puryear J, Cairney J. 1993. A simple and efficient method for isolating RNA from pine trees. *Plant Molecular Biology Reporter* **11**: 113–116.

Cheung AY, Wu HM. 2011. THESEUS 1, FERONIA and relatives: a family of cell wall-sensing receptor kinases? *Current Opinion in Plant Biology* **14**: 632–641.

Ciftci-Yilmaz S, Mittler R. 2008. The zinc finger network of plants. *Cellular and Molecular Life Sciences* **65**: 1150–1160.

Ciftci-Yilmaz S, Morsy MR, Song LH, Coutu A, Krizek BA, Lewis MW, Warren D, Cushman J, Connolly EL, Mittler R. 2007. The EAR-motif of the Cys2/His2-type zinc finger protein ZAT7 plays a key role in the defense response of *Arabidopsis* to salinity stress. *Journal of Biological Chemistry* **282**: 9260–9268.

Coutand C, Dupraz C, Jaouen G, Ploquin S, Adam B. 2008. Mechanical stimuli regulate the allocation of biomass in trees: demonstration with young *Prunus avium* trees. *Annals of Botany* **101**: 1421–1432.

Coutand C, Julien JL, Moulia B, Mauget JC, Guitard D. 2000. Biomechanical study of the effect of a controlled bending on tomato stem elongation: global mechanical analysis. *Journal of Experimental Botany* **51**: 1813–1824.

Coutand C, Martin L, Leblanc-Fournier N, Decourteix M, Julien JL, Moulia B. 2009. Strain mechanosensing quantitatively controls diameter growth and the level of expression of the *PtaZFP2* mechanosensitive gene in poplar. *Plant Physiology* **151**: 223–232.

Coutand C, Moulia B. 2000. Biomechanical study of the effect of a controlled bending on tomato stem elongation: local strain sensing and spatial integration of the signal. *Journal of Experimental Botany* **51**: 1825–1842.

Curtis MD, Grossniklaus U. 2003. A gateway cloning vector set for high-throughput functional analysis of genes in planta. *Plant Physiology* **133**: 462–469.

Davletova S, Schlauch K, Coutu J, Mittler R. 2005. The zinc-finger protein Zat12 plays a central role in reactive oxygen and abiotic stress signaling in Arabidopsis. *Plant Physiology* **139**: 847–856.

Del Bem LE, Vincentz MG. 2010. Evolution of xyloglucan-related genes in green plants. *BMC Evolutionary Biology* **10**: 341.

Devaiah BN, Nagarajan VK, Raghothama KG. 2007. Phosphate homeostasis and root development in Arabidopsis are synchronized by the zinc finger transcription factor ZAT6. *Plant Physiology* **145**: 147–159.

Englbrecht CC, Schoof H, Bohm S. 2004. Conservation, diversification and expansion of C2H2 zinc finger proteins in the *Arabidopsis thaliana* genome. *BMC Genomics* **5**: 39.

Etchells JP, Turner SR. 2010. Orientation of vascular cell divisions in Arabidopsis. *Plant Signaling and Behavior* **5**: 730–732.

Ge Y, Dudoit S, Speed T. 2003. Resampling-based multiple testing for microarray data analysis. *TEST* **12**: 1–77.

Gourcilleau D, Lenne C, Armenise C, Moulia B, Julien J-L, Bronner G, Leblanc-Fournier N. 2011. Phylogenetic study of plant Q-type C2H2 zinc finger proteins and expression analysis of poplar genes in response to osmotic, cold and mechanical stresses. *DNA Research* **18**: 77–92.

Guo H, Li L, Ye H, Yu X, Algreen A, Yin Y. 2009. Three related receptor-like kinases are required for optimal cell elongation in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences, USA* **106**: 7648–7653.

Hamant O. 2013. Widespread mechanosensing controls the structure behind the architecture in plants. *Current Opinion in Plant Biology* **16**: 654–660.

Hamant O, Heisler MG, Jonsson H, Krupinski P, Uyttewaal M, Bokov P, Corson F, Sahlin P, Boudaoud A, Meyerowitz EM *et al.* 2008. Developmental patterning by mechanical signals in Arabidopsis. *Science* **322**: 1650–1655.

Hermann M, Maier F, Masroor A, Hirth S, Pfitzner AJ, Pfitzner UM. 2013. The Arabidopsis NIMIN proteins affect NPR1 differentially. *Frontiers in Plant Science* **4**: 88.

Humphrey TV, Bonetta DT, Goring DR. 2007. Sentinels at the wall: cell wall receptors and sensors. *New Phytologist* **176**: 7–21.

Ingber DE. 2005. Mechanical control of tissue growth: function follows form. *Proceedings of the National Academy of Sciences, USA* **102**: 11571–11572.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. 2003. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics* **4**: 249–264.

Jaffe MJ. 1973. Thigmomorphogenesis. The response of plant growth and development to mechanical stimulation. *Planta* **114**: 143–157.

Kazan K. 2006. Negative regulation of defense and stress genes by EAR motif containing repressors. *Trends in Plant Science* **11**: 109–112.

Kern KA, Ewers FW, Telewski FW, Koehler L. 2005. Mechanical perturbation affects conductivity, mechanical properties and aboveground biomass of hybrid poplars. *Tree Physiology* **25**: 1243–1251.

Kilian J, Whitehead D, Horak J, Wanke D, Weinl S, Batistic O, D'Angelo C, Bornberg-Bauer E, Kudla J, Harter K. 2007. The AtGenExpress global stress expression data set: protocols, evaluation and model data analysis of UV-B light, drought and cold stress responses. *Plant Journal* **50**: 347–363.

Knight MR, Smith SM, Trewavas AJ. 1992. Wind-induced plant motion immediately increases cytosolic calcium. *Proceedings of the National Academy of Sciences, USA* **89**: 4967–4971.

Kodaira KS, Qin F, Tran LS, Maruyama K, Kidokoro S, Fujita Y, Shinozaki K, Yamaguchi-Shinozaki K. 2011. Arabidopsis Cys2/His2 zinc-finger proteins AZF1 and AZF2 negatively regulate abscisic acid-repressive and auxin-inducible genes under abiotic stress conditions. *Plant Physiology* **157**: 742–756.

Kubo K, Sakamoto A, Kobayashi A, Rybka Z, Kanno Y, Nakagawa H, Nishino T, Takatsuji H. 1998. Cys(2)/His(2) zinc-finger protein family of petunia: evolution and general mechanism of target-sequence recognition. *Nucleic Acids Research* **26**: 608–615.

Leblanc-Fournier N, Coutand C, Crouzet J, Brunel N, Lenne C, Moulia B, Julien JL. 2008. *Jr-ZFP2*, encoding a Cys2/His2-type transcription factor, is

involved in the early stages of the mechano-perception pathway and specifically expressed in mechanically stimulated tissues in woody plants. *Plant, Cell & Environment* **31**: 715–726.

Lee D, Polisensky DH, Braam J. 2005. Genome-wide identification of touch- and darkness-regulated Arabidopsis genes: a focus on calmodulin-like and XTH genes. *New Phytologist* **165**: 429–444.

Leplé JC, Brasileiro ACM, Michel MF, Delmotte F, Jouanin L. 1992. Transgenic poplars – expression of chimeric genes using 4 different constructs. *Plant Cell Reports* **11**: 137–141.

Love J, Björklund S, Vahala J, Hertzberg M, Kangasjärvi J, Sundberg B. 2009. Ethylene is an endogenous stimulator of cell division in the cambial meristem of *Populus*. *Proceedings of the National Academy of Sciences, USA* **106**: 5984–5989.

Luo X, Bai X, Zhu D, Li Y, Ji W, Cai H, Wu J, Liu B, Zhu Y. 2012. GsZFP1, a new Cys2/His2-type zinc-finger protein, is a positive regulator of plant tolerance to cold and drought stress. *Planta* **235**: 1141–1155.

Martin L, Leblanc-Fournier N, Azri W, Lenne C, Henry C, Coutand C, Julien JL. 2009. Characterization and expression analysis under bending and other abiotic factors of *PtaZFP2*, a poplar gene encoding a Cys2/His2 zinc finger protein. *Tree Physiology* **29**: 125–136.

Martin L, Leblanc-Fournier N, Julien JL, Moulia B, Coutand C. 2010. Acclimation kinetics of physiological and molecular responses of plants to multiple mechanical loadings. *Journal of Experimental Botany* **61**: 2403–2412.

McMaugh SJ, Lyon BR. 2003. Real-time quantitative RT-PCR assay of gene expression in plant roots during fungal pathogenesis. *BioTechniques* **34**: 982–986.

Monshausen GB, Bibikova TN, Weisenseel MH, Gilroy S. 2009. Ca^{2+} regulates reactive oxygen species production and pH during mechanosensing in *Arabidopsis* roots. *Plant Cell* **21**: 2341–2356.

Monshausen GB, Gilroy S. 2009. Feeling green: mechanosensing in plants. *Trends in Cell Biology* **19**: 228–235.

Monshausen GB, Haswell ES. 2013. A force of nature: molecular mechanisms of mechanoperception in plants. *Journal of Experimental Botany* **64**: 4663–4680.

Morizet J, Mingeau M. 1976. Effect of environment on water-uptake, as studied on beheaded exudating tomato. 1. Role of nutrients. *Annales Agronomiques* **27**: 183–205.

Moulia B, Coutand C, Lenne C. 2006. Posture control and skeletal mechanical acclimation in terrestrial plants: implications for mechanical modeling of plant architecture. *American Journal of Botany* **93**: 1477–1489.

Moulia B, Der Loughian C, Bastien R, Martin L, Rodríguez M, Gourcilleau D, Barbacci A, Badel E, Franchel J, Lenne C *et al.* 2011. Integrative mechanobiology of growth and architectural development in changing mechanical environments. In: Wojtaszek P, ed. *Mechanical integration of plant cells and plants. Springer, series: signaling and communication in plants*. Berlin Heidelberg, Germany: Springer-Verlag GmbH, 269–302.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473–492.

Nilsson J, Karlberg A, Antti H, Lopez-Vernaza M, Mellerowicz E, Perrot-Rechenmann C, Sandberg G, Bhalerao RP. 2008. Dissecting the molecular basis of the regulation of wood formation by auxin in hybrid aspen. *Plant Cell* **20**: 843–855.

Nishikubo N, Takahashi J, Roos AA, Derba-Maceluch M, Piens K, Brumer H, Teeri TT, Stålbrand H, Mellerowicz EJ. 2011. Xyloglucan endo-transglycosylase-mediated xyloglucan rearrangements in developing wood of hybrid aspen. *Plant Physiology* **155**: 399–413.

Ohta M, Matsui K, Hiratsu K, Shinshi H, Ohme Takagi M. 2001. Repression domains of class II ERF transcriptional repressors share an essential motif for active repression. *Plant Cell* **13**: 1959–1968.

Potters G, Pasternak TP, Guisez Y, Jansen MA. 2009. Different stresses, similar morphogenic responses: integrating a plethora of pathways. *Plant, Cell & Environment* **32**: 158–169.

Potters G, Pasternak TP, Guisez Y, Palme KJ, Jansen MA. 2007. Stress-induced morphogenic responses: growing out of trouble? *Trends in Plant Science* **12**: 98–105.

Pruyn ML, Ewers BJ, Telewski FW. 2000. Thigmomorphogenesis: changes in the morphology and mechanical properties of two *Populus* hybrids in response to mechanical perturbation. *Tree Physiology* **20**: 535–540.

Rizhsky L, Davletova S, Liang H, Mittler R. 2004. The zinc finger protein Zat12 is required for cytosolic ascorbate peroxidase 1 expression during oxidative stress in *Arabidopsis*. *Journal of Biological Chemistry* **279**: 11736–11743.

Rodriguez M, de Langre E, Moulia B. 2008. A scaling law for the effects of architecture and allometry on tree vibration modes suggests a biological tuning to modal compartmentalization. *American Journal of Botany* **95**: 1523–1537.

Rushton PJ, Somssich IE, Ringler P, Shen QJ. 2010. WRKY transcription factors. *Trends in Plant Science* **15**: 247–258.

Sakamoto H, Maruyama K, Sakuma Y, Meshi T, Iwabuchi M, Shinozaki K, Yamaguchi-Shinozaki K. 2004. *Arabidopsis* Cys2/His2-type zinc-finger proteins function as transcription repressors under drought, cold, and high-salinity stress conditions. *Plant Physiology* **136**: 2734–2746.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**: 671–675.

Sistrunk ML, Antosiewicz DM, Purugganan MM, Braam J. 1994. Arabidopsis Tch3 encodes a novel Ca^{2+} binding-protein and shows environmentally-induced and tissue-specific regulation. *Plant Cell* **6**: 1553–1565.

Stull RB. 1988. *An introduction to boundary layer meteorology*. Dordrecht, the Netherlands: Kluwer Academic Publishers.

Sun SJ, Guo SQ, Yang X, Bao YM, Tang HJ, Sun H, Huang J, Zhang HS. 2010. Functional analysis of a novel Cys2/His2-type zinc finger protein involved in salt tolerance in rice. *Journal of Experimental Botany* **61**: 2807–2818.

Telewski FW. 2006. A unified hypothesis of mechanoperception in plants. *American Journal of Botany* **93**: 1466–1476.

Telewski FW, Jaffe MJ. 1986. Thigmomorphogenesis: field and laboratory studies of *Abies fraseri* in response to wind or mechanical perturbation. *Physiologia Plantarum* **66**: 211–218.

Telewski FW, Pruyn M. 1998. Thigmomorphogenesis: a dose response to flexing in *Ulmus americana* seedlings. *Tree Physiology* **18**: 65–68.

Vadassery J, Reichelt M, Hause B, Gershenzon J, Boland W, Mithöfer A. 2012. CML42-mediated calcium signaling coordinates responses to Spodoptera herbivory and abiotic stresses in Arabidopsis. *Plant Physiology* **159**: 1159–1175.

Vandenbussche F, Vaseva I, Vissenberg K, Van Der Straeten D. 2012. Ethylene in vegetative development: a tale with a riddle. *New Phytologist* **194**: 895–909.

Vanzin GF, Madson M, Carpita NC, Raikhel NV, Keegstra K, Reiter WD. 2002. The *mur2* mutant of *Arabidopsis thaliana* lacks fucosylated xyloglucan because of a lesion in fucosyltransferase AtFUT1. *Proceedings of the National Academy of Sciences, USA* **99**: 3340–3345.

Voelker SL, Lachenbruch B, Meinzer FC, Strauss SH. 2011. Reduced wood stiffness and strength, and altered stem form, in young antisense 4CL transgenic poplars with reduced lignin contents. *New Phytologist* **1894**: 1096–1109.

Vogel JT, Zarka DG, Van Buskirk HA, Fowler SG, Thomashow MF. 2005. Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. *Plant Journal* **41**: 195–211.

Xu W, Purugganan MM, Polisensky DH, Antosiewicz DM, Fry SC, Braam J. 1995. *Arabidopsis TCH4*, regulated by hormones and the environment, encodes a xyloglucan endotransglycosylase. *Plant Cell* **7**: 1555–1567.

Xu X, Chen C, Fan B, Chen Z. 2006. Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors. *Plant Cell* **18**: 1310–1326.

Zuo JR, Niu QW, Chua NH. 2000. An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. *Plant Journal* **24**: 265–273.

Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Details on transgene construction, Southern blot analysis and levels of *PtaZFP2* expression without 17β-estradiol in *PtaZFP2*-OE (#30 and #39) and WT poplars.

Fig. S2 Schematic representation of the stress vs strain curves during a three-point bending test.

Table S1 Sequences of primers used for qRT-PCR

Table S2 List of up- and down-regulated genes in *PtaZFP2*-OE poplars

Table S3 List of over-represented GO terms in the *Arabidopsis* matches of the differentially expressed genes in *PtaZFP2*-OE poplars

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.